

Characterization of *Pseudomonas aeruginosa* from cystic fibrosis lung infections

Introduction

P. aeruginosa is an opportunistic pathogen that causes a range of acute diseases in both humans and animals due to its large number of virulence genes which directly interfere with different hosts and their defence systems. One of the best studied human infections caused by *P. aeruginosa* is airway infections in patients suffering from the genetic disorder cystic fibrosis (CF). Due to a severely reduced mechanical clearing of the airways in these patients they acquire multiple infections of various bacteria and fungi, but the major cause of morbidity and mortality is chronic infection of *P. aeruginosa* in the lungs. The development of chronic lung infections follows a characteristic pattern (Figure 1). Typically, a period of intermittent colonization with *P. aeruginosa* early in the life of the patient precedes the establishment of chronic infections. These early-infecting strains typically resemble those found in the environment. This stage is temporary, and eventually the airways of nearly all patients become permanently colonized by *P. aeruginosa*. Once established, these chronic lung infections are difficult to eradicate even with intensive antibiotic treatment.

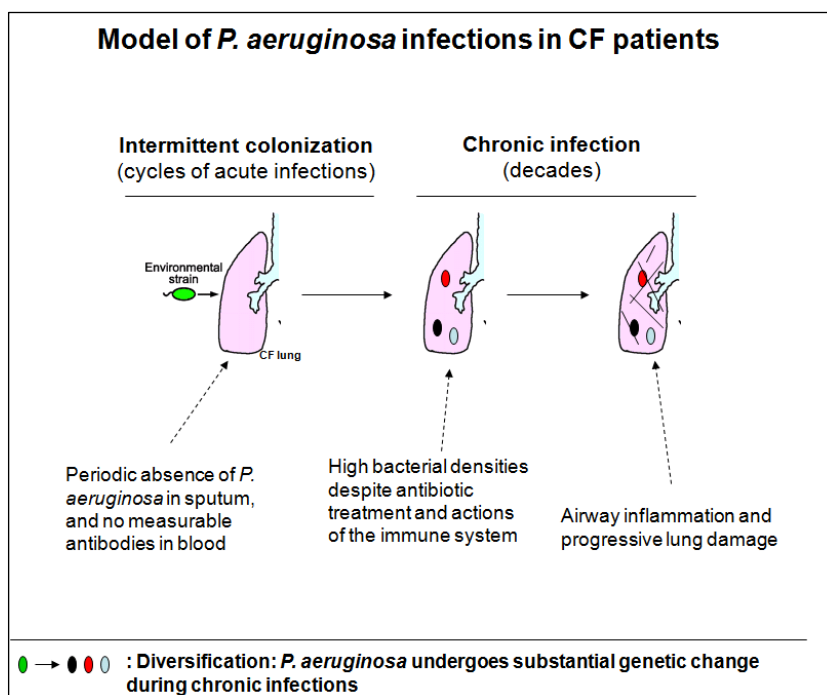


Figure 1. Model of *Pseudomonas aeruginosa* infections in cystic fibrosis patients.

At the molecular level, the development of chronic infections have been described as multi-stage colonization processes which initially require the encoded virulence properties of the bacteria to establish a successful colonization followed by switching to a less virulent expression mode controlled global transcriptional regulators in *P. aeruginosa*, which direct the bacterial population towards a chronic infection. At the same time, the infection process in the CF airways is associated with extensive genetic adaptation and microevolution of the infecting bacteria: During the long-term chronic infection stage the bacteria gradually lose the function of many genes associated with bacterial pathogenicity. The accumulation of mutations results in genetic variants with

phenotypes of which many are not usually observed among environmental isolates. These phenotypes include loss of motility, loss of effector proteins of the type III secretion system, reduced virulence, and increased antibiotic resistance. In some cases, specific genes have been found to be hot spots for mutations during CF infections. These common targets include *lasR*, which encodes a quorum-sensing regulator, and *mucA*, which results in the overproduction of alginate and conversion to the frequently found mucoid phenotype.

Description of the exercise

As a part of our research on *P. aeruginosa* infections, we have been following various CF patient groups and their infecting *P. aeruginosa* strains for several years (See supplementary papers).

In this exercise, each team will analyze 2 isolates of *P. aeruginosa* strains sampled from a CF patient that is of particular interest to us. All isolates that we will analyze in this exercise are from the same host but different years (longitudinal samples). We know very little of the isolates but on the basis of genotyping and relevant phenotype assays performed on these isolates each group will make new contributions to our understanding of the infection dynamics in this particular patient.

Experimental keywords: genotyping using SNP arrays, infection dynamics analysis, antibiotic resistance profiling.

Relevant references:

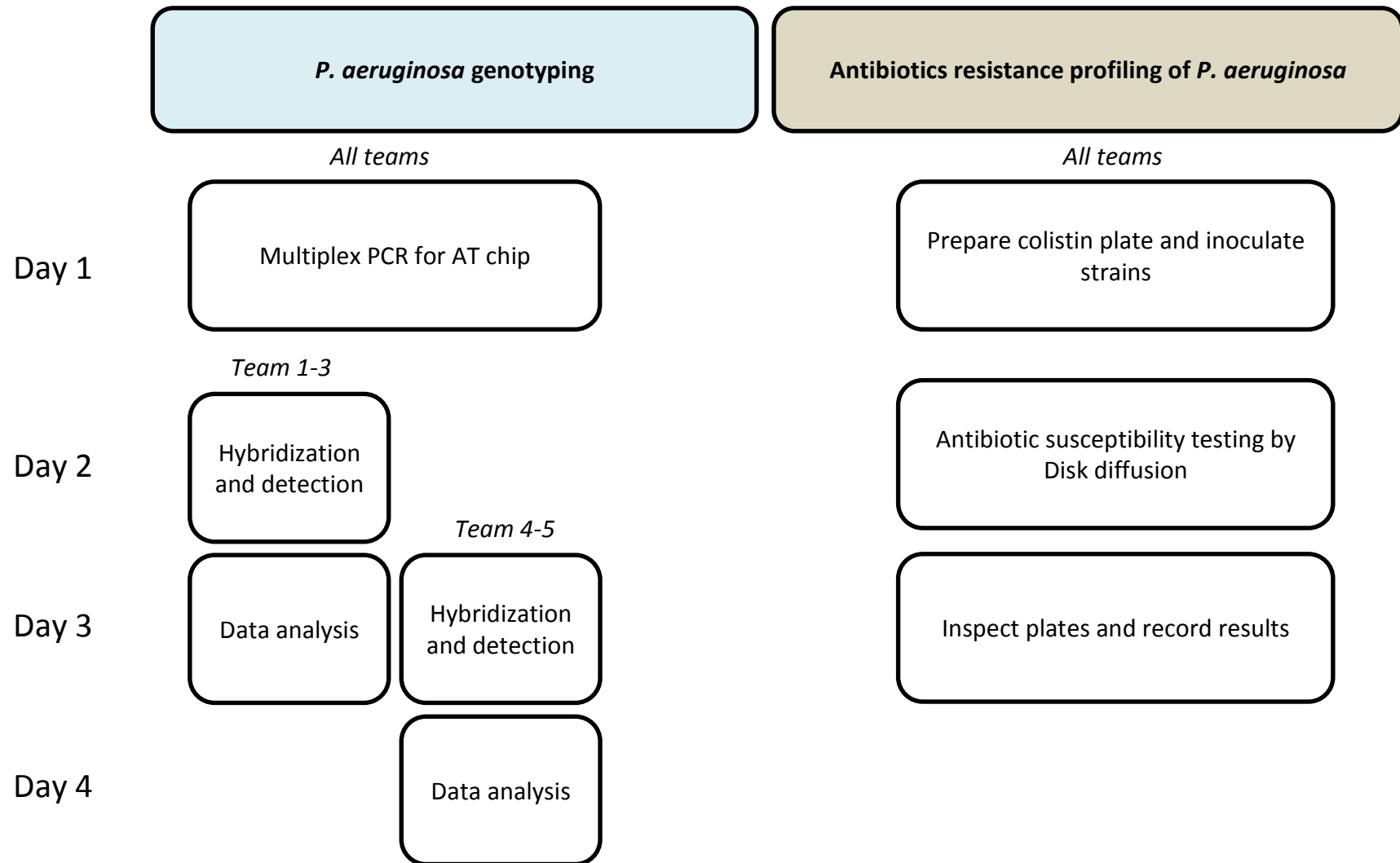
Lars Jelsbak, Helle Krogh Johansen, Anne-Louise Frost, Regitze Thøgersen, Line Elnif Thomsen, Oana Ciofu, Lei Yang, Janus Haagenzen, Niels Høiby, Søren Molin. (2007). ***Molecular epidemiology and dynamics of Pseudomonas aeruginosa populations in lungs of cystic fibrosis patients.*** Infect Immun. 75(5):2214-24.

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Wiehlmann L, Wagner G, Cramer N, Siebert B, Gudowius P, Morales G, Köhler T, van Delden C, Weinl C, Slickers P, Tümmler B. (2007) ***Population structure of Pseudomonas aeruginosa.*** Proc Natl Acad Sci U S A. 104(19):8101-6.

Overview of the experimental procedures



Experimental procedures

Day 1.

(A) *P. aeruginosa* genotyping

It is important that you have an understanding of the ArrayTube genotyping method before we start the experiments. Please consult appendix 3 and also the supplementary papers for this exercise – in particular the paper by Wiehlmann et al (2007).

Note: AT chip genotyping is performed on both isolates in independent reactions. The following protocol is described for 1 reaction

Preparation of bacteria

1. Swipe 2 loops of bacteria off the agar plate and resuspend in 100µl H₂O in an eppendorf tube.
2. Heat for 5min in a heating block at 99C
3. Cool on ice before using 5µl of the suspension for your PCR reaction (see next step)

Preparation of the PCR reaction:

1. Prepare a Master Mix by combining 4.9 µl of B1 (2X Labelling buffer) and 0.1 µl of B2 (DNA polymerase) per specimen.
2. Mix the following in a PCR tube:
5 µl DNA suspension
5 µl Master Mix.

2. Amplification protocol:

Initial denaturation 96°C 300 s	
50 cycles with	20 sec at 62°C
	40 sec at 72°C
	60 sec at 96°C
Cool down and store at 4°C	

(B) Antibiotic susceptibility testing using Disk Diffusion

Preparation for tomorrow:

1. Inoculate a single colony of each of the 2 isolate in 10ml LB medium and incubate at 37C ON.
2. Inoculate a single colony of *Pseudomonas aeruginosa* PA01 (reference strain) in 10ml LB medium and incubate at 37C ON

3. We need to prepare colistin plates for “Antibiotic susceptibility testing using Disk Diffusion” tomorrow (Read appendix 1 to learn why):
- Label an LB agar plate for each of your isolates
 - Place a colistin tab (“CO150”) on each plate using forceps
 - After 2 hours at room temperature the tablet is removed (by knocking the plate against the table) and the plate is maintained at room temperature for further 18 or 22 hours (overnight).
 - Outline the position of the tab with a pen on the backside of the agar plate.

Day 2.

(A) *P. aeruginosa* genotyping

****Important notes – read the following before you start the experiment***:*

- Do not centrifuge ArrayTubes (AT)
- Do not scratch or touch the array surface (e.g. during pipetting steps with the pipette tip)
- Avoid formation of air bubbles and remove them, if necessary, by mixing with a pipette
- Never let the AT run dry

Preparation of the hybridisation mixture

Prior to hybridization, the AT array needs to be conditioned:

1. Pre-heat the thermomixer to 60°C
2. Add 90 µl of buffer C1 to each PCR tube, mix gently (vigorous mixing results in foaming) and put aside.

Pre-washing of the arrays (2 washing steps)

1. Remove the ArrayTube from the bag
2. Add 500 µl of ultrapure water to each tube
3. Incubate in the thermomixer at 60°C, 550 rpm for 2 minutes
4. Remove and discard the water WITHOUT TOUCHING THE ARRAY SURFACE
5. Add 200 µl buffer C1 to each tube
6. Incubate in the thermomixer at 60°C, 550 rpm for 2 minutes
7. Remove and discard buffer C1
8. Proceed promptly (hybridisation mixtures must be ready when C1 is removed)

Hybridization.

1. Transfer each hybridisation mixture (100 µl) to a prepared ArrayTube (avoid extensive foaming)
2. Incubate for one hour at 60°C, 550 rpm on the thermomixer

Meanwhile start computer, AT Reader and IconoClust-AT recording software.

Washing after Hybridisation

1. Remove the ArrayTubes from the thermomixer and set the thermomixer to 30°C for the following steps.
2. Remove *hybridization mix* from the AT as completely as possible (without toughing the surface)
3. Add 500 µl of buffer C2
4. Remove and discard the washing solution
5. Add 500 µl of buffer C2 and incubate in the thermomixer for 5 min at 30°C, 550 rpm
6. Remove the buffer and repeat the last washing step (step 5).

Staining and detection.

1. Combine reagent C3 (HRP) and buffer C4 = 1: 100 => C3/C4
For one sample mix 1 µl C3 with 100 µl C4.
2. Add 100 µl of C3/C4 to each tube
3. Incubate for 10 min at 30°C and 550 rpm on the thermomixer
4. Remove and discard C3/C4
5. Add 500 µl of buffer C5
6. Remove and discard and repeat step.

Image acquisition

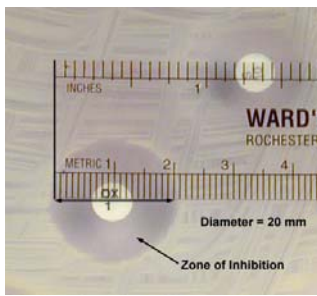
1. Add 100 µl of reagent D1. Immediately insert the AT inside the AT reader and start image acquisition (note: the TMB-staining process is usually finished after 10 min).

Notes:

- Take more than just 1 image.
- The outside of the AT may be gently (!) cleaned using a piece of lens-paper with ethanol
- If there is "dirt" inside the AT, then remove the TMB solution (and the dirt) after you are done with the image acquisition, add 100µl of hybridization solution and acquire an extra image.

(B) Antibiotic susceptibility testing using Disk Diffusion

Susceptibility testing is of great importance to the hospital physician and the practitioner in the treatment of infectious diseases. Knowledge of susceptibility to different antibiotics of an infectious agent facilitates the choice of the most effective antibacterial compounds. In most clinical laboratories the *disk diffusion method* is used routinely for testing the susceptibility of bacterial pathogens. In this part of the exercise, you will test the susceptibility of your isolates to different clinically relevant antibiotics: Gentamycin, Enrofloxacin, and Polymyxins (*a.k.a.* colistin) (see the table below for the disks used in this exercise).



An example of antibiotic susceptibility testing using the Disk Diffusion method. You measure the diameter of zones of growth inhibition in millimeters. The zone seen in this example measures 20 mm in diameter.

Product	Code	Potency
Gentamycin	GEN40	40 µg
Gentamycin	GN250	250 µg
Enrofloxacin	ENROF	10 µg
Polymyxins (Colistin)	CO150	150 µg

1. Dry 1 LB plate per culture for 30min at 37°
2. Dilute each ON culture 10.000x in 0.9% NaCl and spread 100µl on an LB agar plate using a sterile "drigalski spatel".
3. Place the 3 tabs on the agar plates using forceps
4. Incubate the plates at 35C ON.

Day 3:

(A) *P. aeruginosa* genotyping

Data analysis

For the data analysis you need the following items:

- The acquired images from your AT experiment
- The document "Short manual for analysis of AT chip data" (Appendix 5)
- The Excel sheet "AT chip skabelon" which can be found at the course web-site: *Supplementary Manuals/AT chip manuals and data analysis.*

At the course website you may also find an example of the data analysis

(B) Antibiotic susceptibility testing using Disk Diffusion

Inspect plates and record results in the data sheets on the following pages.

Group:

Results: Antibiotic susceptibility testing using Disk Diffusion

Name or number of Clinical isolate	GEN40		GN150		ENROF		CO150	
	Measured Zone diameter in mm	S, I, or R ? ⁽¹⁾	Measured Zone diameter in mm	S, I, or R ? ⁽¹⁾	Measured Zone diameter in mm	S, I, or R ? ⁽¹⁾	Measured Zone diameter in mm	S, I, or R ? ⁽¹⁾

Control:

PAO1								
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⁽¹⁾ The isolate is **S**(sensitive), **I**(Intermediate) or **R**(resistant) toward the tested antibiotics. See “Zone diameter Interpretation Table” in appendix 2.

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NEO-SENSITABS™
Performance of Susceptibility
Testing

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Prediffusion method

Pre-diffusion Method (2 + 18 or 2+22 hours) for Antimicrobials Diffusing Poorly on Agar

High molecular weight antimicrobials (vancomycin, teicoplanin, daptomycin, colistin) diffuse poorly on agar media, resulting in difficult to interpret results when using the disc diffusion method. Rosco Diagnostica has developed a 2 + 18 or 2+22 hour prediffusion technique, permitting an easier differentiation between susceptible and resistant strains when testing against these antimicrobials.

Procedure

One Neo-Sensitabs of the antimicrobial to be tested is placed on an uninoculated plate containing the susceptibility test medium (Mueller-Hinton plain or BHI Agar + 5 % blood). After 2 hours at room temperature ($\leq 25^{\circ}\text{C}$) the tablet (disc) is removed (by knocking the plate against the table) and the plate is maintained at room temperature for further 18 or 22 hours (overnight).

The plate is now inoculated with the strain to be tested using a McFarland 0.5 inoculum.

Additional antibiotic discs (Neo-Sensitabs) may be added now using a dispenser (if MH agar is used) and thereafter the plate is incubated overnight at 35-37 °C.

The zones of inhibition are then measured. Zone breakpoints are tentative and for research use only.

Notice: In the laboratory, the prediffusion plate can be prepared the day before it is inoculated, in which case there is no loss of time and results are obtained within 24 hours.

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Interpretation Zones and MIC Breakpoints according to CLSI

P. aeruginosa, *Acinetobacter* spp., *B. cepacia*, *S. maltophilia*

PSEUDOMONAS AERUGINOSA

Zone diameter interpretative criteria and MIC breakpoints according to CLSI (formerly NCCLS) (1) when testing *P. aeruginosa*, *Acinetobacter* spp., *S. maltophilia*, *B. cepacia*, are listed in the tables below.

Table 3.2-1 Interpretation for *Pseudomonas aeruginosa*

Mueller-Hinton Agar. Inoculum McFarland 0.5. Incubation at 35 °C ± 2 degrees ambient air for 16-18 hours.

NEO-SENSITABS	POTENCY	CODE	Zone diameter in mm			Break-points MIC µg/ml	
			S	I	R	S	R
a)	Amikacin 30 µg	AMI30	≥ 17	16-15	≤ 14	≤ 16	≥ 32
	Aztreonam 30 µg	AZT30	≥ 22	21-16	≤ 15	≤ 8	≥ 32
	Cefepime 30 µg	FEP30	≥ 18	17-15	≤ 14	≤ 8	≥ 32
d)	Cefepime+Clavulanate FEP+C	30+10 µg	detection of ESBL				
d)	Ceftazidime 30 µg	CAZ30	≥ 18	17-15	≤ 14	≤ 8	≥ 32
d)	Ceftazidime+Clavulanate CAZ+C	30+10µg	detection of ESBL				
	Ciprofloxacin 5 µg	CIPR5	≥ 21	20-16	≤ 15	≤ 1	≥ 4
c)	Colistin 10 µg	Co.10					
c)	2+18 hours' prediffusion method		≥ 15	14-11	≤ 10	≤ 2	≥ 8
	Doripenem 10 µg	DOR10	≥ 24	-	-	≤ 2	-
	Fosfomicin 200 µg	FO200	≥ 22	21-19	≤ 18	≤ 16	≥ 32
	Gatifloxacin 5 µg	GATIF	≥ 18	17-15	≤ 14	≤ 2	≥ 8
a)	Gentamicin 10 µg	GEN10	≥ 15	14-13	≤ 12	≤ 4	≥ 8
e) *)	Imipenem 10 µg	IMI10	≥ 22	21-18	≤ 17	≤ 4	≥ 16 per 30/4 2008
b)	Imipenem+EDTA 10+750µg	IM10E	detection of metallo-β-lactamases				
	Levofloxacin 5 µg	LEVOF	≥ 17	16-14	≤ 13	≤ 2	≥ 8
e) *)	Meropenem 10 µg	MRP10	≥ 22	21-18	≤ 17	≤ 4	≥ 16
	Minocycline 30 µg	MIN30	≥ 19	18-15	≤ 14	≤ 4	≥ 16
	Netilmicin 30 µg	NET30	≥ 15	14-13	≤ 12	≤ 4	≥ 8
	Ofloxacin (U) 5 µg	OFL5	≥ 16	15-13	≤ 12	≤ 2	≥ 8
	Piperacillin 100 µg	PIPR	≥ 18	-	≤ 17	≤ 64	≥ 128
	Piperacillin+Tazobactam 100+10µg	PI+TZ	≥ 18	-	≤ 17	≤ 64/4	≤ 128/4
	Tetracyclines 30 µg	TET30	≥ 19	18-15	≤ 14	≤ 4	≥ 16
	Ticarcillin 75 µg	TIC75	≥ 15	-	≤ 14	≤ 64	≥ 128
	Ticarcillin+Clavulanate 75+10 µg	TIM85	≥ 15	-	≤ 14	≤ 64/2	≥ 128/2
a)	Tobramycin 10 µg	TOB10	≥ 15	14-13	≤ 12	≤ 4	≥ 8
	Dipicolinic acid 250 µg	D.P.A	Detection of metallo-β-beta lactamases				

- *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis requires incubation up to 24 hours.

*) *P. aeruginosa* low level resistant to meropenem (MIC 8 µg/ml) or Imipenem cannot be detected using the interpretation recommended by the CLSI. We recommend using Rosco's own interpretation.

Clinical isolates of *P. aeruginosa* heterogeneously resistant to carbapenems have been isolated (1). Subcolonies appearing within the zone of inhibition of Imipenem/Meropenem show higher MIC values. Automated systems and conventional agar dilution MICs using the standard 10⁴ CFU per spot inoculum may miss carbapenem-resistant mutants.

Detection of *P. aeruginosa* resistant to Colistin in University Hospital in Barcelona (reference 3).

ArrayTube (AT) chip genotyping overview

We apply molecular genotyping methods to monitor the *Pseudomonas aeruginosa* infection dynamics and epidemiology among CF patients. We primarily use the ArrayTube (AT) microarray from CLONDIAG to discriminate between clones isolated from infected patients. The AT technology is a DNA-chip based genotyping method in which labelled DNA is directly generated from a bacterial colony by multiplex PCR and then hybridized onto a microarray to yield a binary marker genotype (see Figure 1). We also use MLST and PFGE techniques for genotyping.

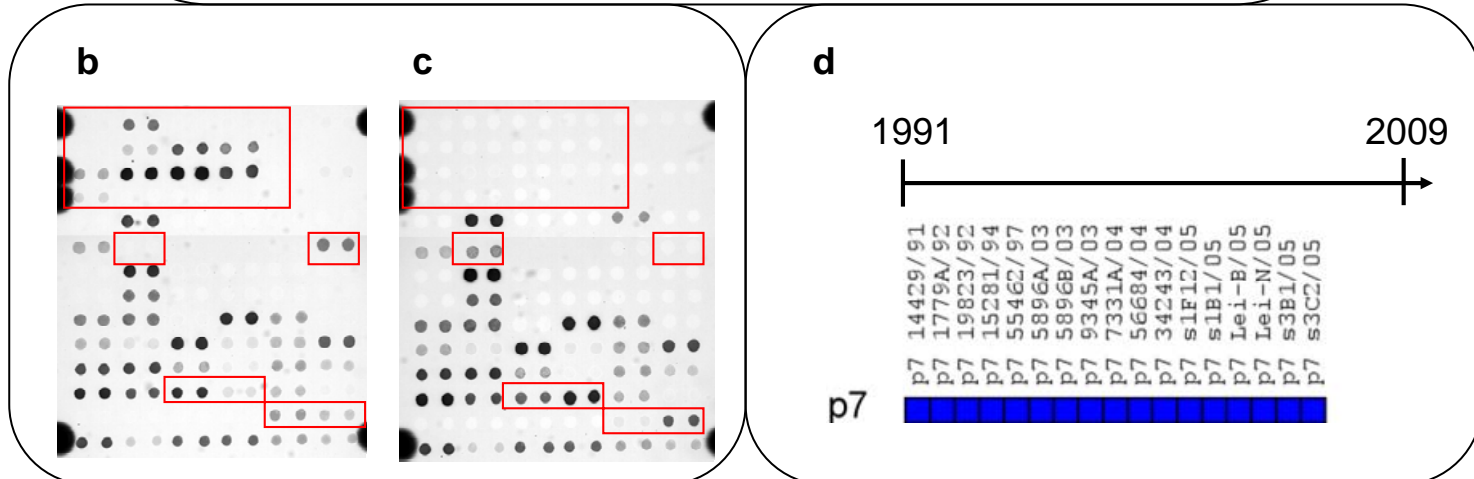
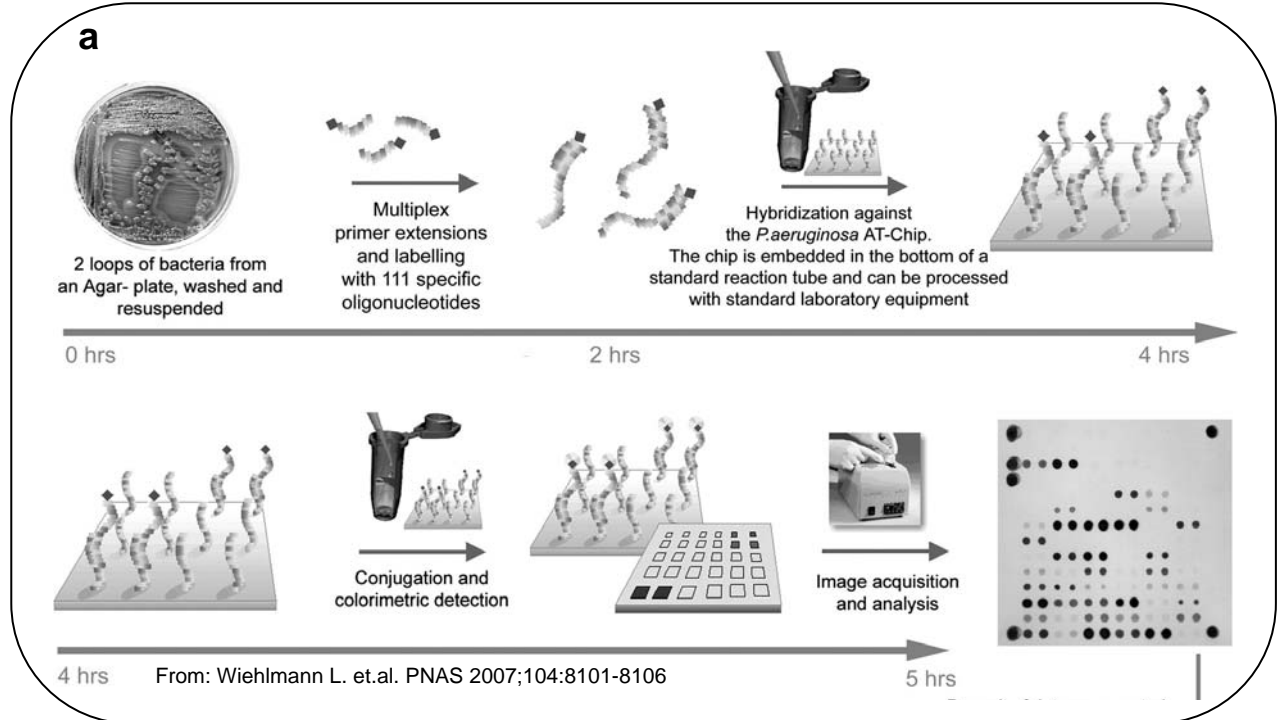


Figure 1. Molecular genotyping of *Pseudomonas aeruginosa*. **(a)** Outline of the experimental protocol for *P. aeruginosa* genotyping with the AT chip. **(b+c)** Examples of 2 different *P. aeruginosa* clones isolated from the same CF patient and genotyped using the AT chip. Red boxes indicate differences in the hybridization pattern. **(d)** *P. aeruginosa* infection dynamics in a single CF patient (patient “p7”) with chronic *P. aeruginosa* lung infection since 1991. Genotyping of stored isolates showed that p7 had been infected with the same clone (indicated by blue squares in the figure) for the entire infection period. For details see Jelsbak et al (2007) Infect Immun. 75:2214-24.

Solutions used in Array Tube (AT) Genotyping of *Pseudomonas aeruginosa*.

DNA labelling and amplification

B1_{Pa}: Labelling Buffer. Store at 2-8°C. Surplus: 40%.

B2: Labelling Enzyme. Store at 2-8°C. Surplus: 200%.

Hybridisation and Detection

ArrayTubes, protected against light and sealed under inert gas. Store at 18°C to 28°C. After opening to be used within two weeks. Protect them against humidity and dust and store them at a dark place. Avoid ANY touching or scratching the microarray on the bottom of the well. CAUTION: Do not store or handle unused tubes above 60% relative humidity since this may irreversibly corrode the spots.

C1: Hybridisation Buffer. Store at 18-28 °C, protect against sunlight. Surplus: 100%.

C2: Washing Buffer 1. Store at 18 °C - 28 °C, protect against direct sunlight. Surplus: 67%.

C3: HRP Conjugate 100x. Store at 2-8 °C, protect against direct sunlight. Surplus: 200%.

C4: Conjugate Buffer. Store at 18°C to 28°C, protect against direct sunlight. Surplus: 500%

C5: Washing Buffer 2. Store at 18°C to 28°C, protect against direct sunlight. Surplus: 140%.

D1: Horseradish Peroxidase Substrate. Store at 2-8°C, protect against direct sunlight. Surplus: 140%.

Short manual for analysis of AT chip data

1. Open the image-file of the AT chip result that you want to analyze: Open the image using e.g. 'picture-viewer'.
2. Make sure the image has the correct orientation: 3 control-spots in upper left corner (see figure 1). If the orientation is wrong it may be corrected by 'flipping' the image.

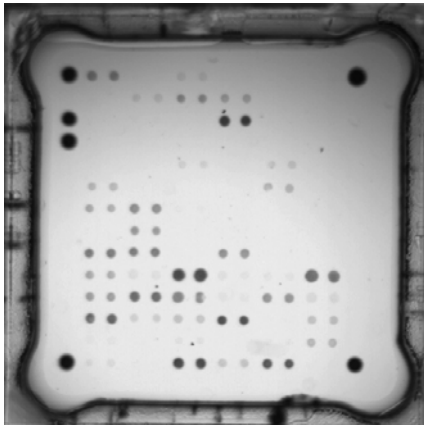


Figure 1. An ArrayTube image. Note the dark control-spots in the corners of the chip.

3. It is a good idea to make a print-out of the image for your files.
4. Print out the schematic outline of the AT chip (Figure 2 – See Figure 3 for a larger version).
5. Focus first on determining the SNP pattern. The SNP spots are found on the lower part of the AT chip (see Figure 3). With the help of the "SNP evaluation" figure (Figure 4) you can now determine whether the SNP are either 'wildtype' or 'mutant'. Record your results with a pen on the outline printout.

NOTE: The 3 SNPs 'oprL', fliCa' and 'oprI' are measured twice on the AT chip.

NOTE: Measurement of the 'fliCa' SNP is only meaningful when there is a signal in the variable gene 'fliCa' further up on the AT chip.

NOTE: If you cannot determine whether it is a mutant or a wildtype signal (maybe there is no signal at all) then note that on the outline printout.

	1	2	3	4	5	6
14	C-45	C-46	C-47	PAGI-3-1	PAGI-3-8	PAGI-2-1
13	PAGI-2/3-1	PAGI-2/3-4	PAGI-2/3-5	PAGI-2/3-6		
12	pKL-1	pKL-3	TB-C47-1	TB-C47-2	PAPI-1-pili-chap.	PAPI-1-LuBiPro
11	pKLC-unknown	pKLC-adhesin	pKLC-metabol.			
10	fpvA type I	fpvA type II a	fpvA type II b	fpvA type III	fpvB	LES
9	PA0636	PA0722			PAGI-1	PAPI-2-PA0980
8	PA0728	PA2185	fla-island-1	fla-2-orfA	47D7-1	PAPI-2-Acyltransf.
7	PA2221	PA3835	fla-2-orfI	fla-2-orfJ	47D7-2	PAPI-2-XF1753
6	wt ampC-7 mut		fliCa	fliCb	exoS	exoU
5	wt ampC-4 mut		wt ampC-6 mut		wt ampC-8 mut	
4	wt oprL (2) mut		wt ampC-1 mut		wt ampC-3 mut	
3	wt crs-1 mut		wt crs-2 mut		wt oprL (1) mut	
2	wt fliCa (1) mut		wt fliCa (2) mut		wt alkB2 mut	
1	wt oriC mut		wt oprL (1) mut		wt oprL (2) mut	

Figure 2. A schematic outline of the AT chip. Note the control spots in the corners. The “SNP” part of the chip is highlighted in red

- Now you focus on the remaining spots (the variable genes). Use the outline printout and evaluate all spot. Simply record if you see a signal (This means that the gene is present). If you do not see a signal, then the gene is not there.

NOTE: Make sure to note any problems with the present/absent determinations

- Collect your data in an electronic form for further analysis

For example, write your data into the Excel workbook called “AT chip skabelon”. This self-explaining workbook is a good place to collect, store and analyze your data.

Write the data into the “Empty data sheet” in this format:

For the SNP data, type: ‘0’ for wildtype, ‘1’ for mutant, ‘-1’ for undetermined.

For the variable genes, type: ‘0’ for absent and ‘1’ for present.

- The color of the cells in the excel sheet will change automatically.
- A hexadecimal code will be calculated for each data-set.
- Remember to write your notes/comments for each data-set
- Use the “cluster” sheet to sort among different data sets.

Want help?!

- Consult this AT chip analysis example:
<http://www.pnas.org/content/suppl/2007/04/18/0609213104.DC1/09213SuppAppendix.pdf>
- This paper describes the development and use of the ArrayTube genotyping method for *P. aeruginosa*: Wiehlmann et al. (2007) Population structure of *Pseudomonas aeruginosa*. PNAS 104(19):8101-6. <http://www.pnas.org/content/104/19/8101.full.pdf>

	1	2	3	4	5	6
14	C-45	C-46	C-47	PAGI-3-1	PAGI-3-8	PAGI-2-1
13	PAGI-2/3-1	PAGI-2/3-4	PAGI-2/3-5	PAGI-2/3-6		
12	pKL-1	pKL-3	TB-C47-1	TB-C47-2	PAPI-1-pili-chap.	PAPI-1-LuBiPro
11	pKLC-unknown	pKLC-adhesin	pKLC-metabol.			
10	fpvA type I	fpvA type II a	fpvA type II b	fpvA type III	fpvB	LES
9	PA0636	PA0722			PAGI-1	PAPI-2-PA0980
8	PA0728	PA2185	fla-island-1	fla-2-orfA	47D7-1	PAPI-2-Acyltransf.
7	PA2221	PA3835	fla-2-orfI	fla-2-orfJ	47D7-2	PAPI-2-XF1753
6	wt ampC-7 mut		fliCa	fliCb	exoS	exoU
5	wt ampC-4 mut		wt ampC-5 mut		wt ampC-6 mut	
4	wt oprI (2) mut		wt ampC-1 mut		wt ampC-3 mut	
3	wt citS-1 mut		wt citS-2 mut		wt oprI (1) mut	
2	wt fliCa (1) mut		wt fliCa (2) mut		wt alkB2 mut	
1	wt oriC mut		wt oprL (1) mut		wt oprL (2) mut	

Figure 3. A schematic outline of the AT chip. Note the control spots in the corners.

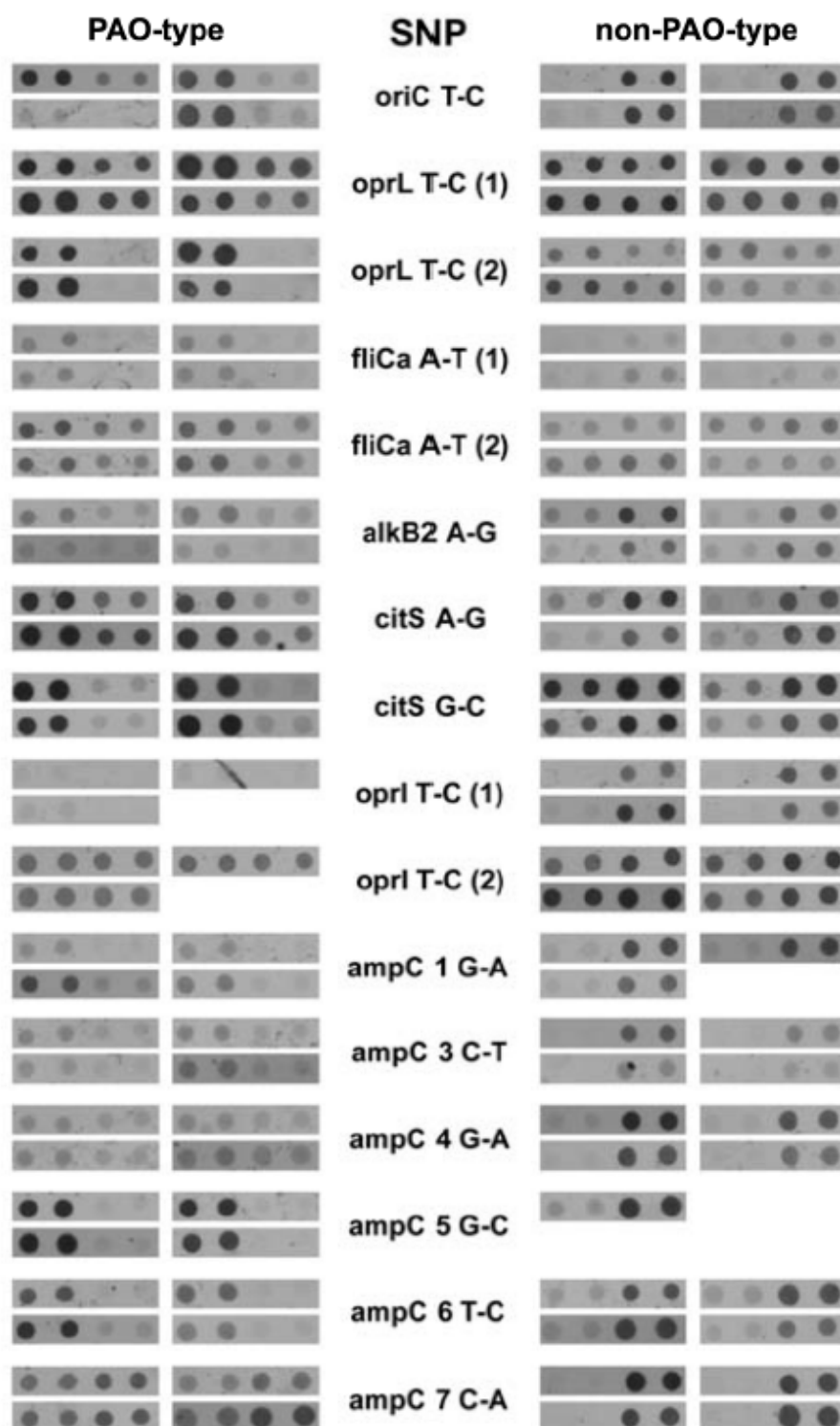


Figure 4. Visual guide for the evaluation of the SNP pattern of the *P. aeruginosa* array tube array. For each SNP, PAO1-type and non-PAO1-type alleles are represented by several sets of primary hybridization data taken from separate strains and experiments